

DNA Aptamer-Based Bioanalysis of IgE by Fluorescence Anisotropy

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A rapid, homogeneous aptamer-based bioanalysis is reported for the sensitive detection of immunoglobulin E (IgE) using fluorescence polarization (FP). 5'-End-labeled D17.4 DNA aptamer was used for IgE detection based on the anisotropy differences of the labeled ligand. Two different fluorophores, fluorescein and Texas Red, were used to analyze IgE in the low-nanomolar range with high specificity. Measurable anisotropy changes were observed with a short equilibration time. Analysis of the binding data reveals a possible cooperative binding process in solution. The nature of the fluorophore clearly influences the sensitivity of the analysis more than the tether length used for the dye conjugation. The local fluorophore motion is seen to influence the sensitivity of the FP probe significantly. Texas Red is seen to be relatively more sensitive for this approach and has apparently favorable dye–DNA interactions, and a limit of detection of 350 pM was obtained. Significant temperature dependence of the FP responses has been observed in this work. Ionic composition of the binding buffer also influences the assay sensitivity. The results confirm the promise and potential of similar homogeneous assays for aptamer-based bioanalysis.

The rapid detection of binding events is essential in drug discovery,¹ diagnostic assays,^{2,3} and, in general, bioanalytical methodologies where biomolecular recognition is important.⁴ In many cases, the binding event is measured by making a separation, typically facilitated by immobilizing the recognition element on a surface, and heterogeneous assays⁵ are based on this principle. However, the demands of high throughput have put greater emphasis on homogeneous assays that do not require separations. Fluorescence polarization (FP) has been used for a

number of years as the basis for clinical diagnostic tests, in particular for immunoassays of therapeutic drugs.^{6,7} The binding of the labeled drug to the antibody restricts motion of the drug, thus altering its FP properties. This approach is, unfortunately, not generally applicable to analysis of proteins because only very small changes in FP can be observed. DNA aptamers,⁸ relatively small molecules to be described below, open up the possibility of employing a small fluorescently labeled biological recognition element to analyze larger molecules.

Combinatorial in vitro ligand selection techniques such as SELEX⁹ have yielded DNA- and RNA-based recognition elements termed “aptamers”. These are short, unique nucleic acid sequences that assume specific secondary structures and orient conformationally to bind cognate targets.¹⁰ The sheer variety of analytes that these nucleic acid reagents can recognize including small molecules, dyes, peptides, proteins, and even cell surfaces has increased the applicability of aptamers as analytical reagents.¹¹ An important advantage of using these DNA or RNA aptamers is the readily available nucleic acid labeling chemistries to incorporate fluorophores and luminophores.¹² DNA aptamers are usually preferred, in comparison to RNA, for their better chemical and enzymatic stability. Furthermore, automated aptamer generation platforms¹³ and improved DNA syntheses have opened up more possibilities for utilizing DNA aptamers in analytical method development. Advances in this area have been made with applications in affinity chromatography, capillary electrochromatography, affinity probe capillary electrophoresis (APCE),^{14–17} array-based protein detection,¹⁸ and biosensors.^{19–21} Sensitive protein detection

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has been accomplished by using DNA aptamers in both homogeneous and heterogeneous formats.^{22,23} In the area of homogeneous assays using DNA aptamers, the adenosine aptamer labeled with fluorescein or acridine has been utilized to detect ATP based on fluorescence intensity changes observed upon the binding process.²⁴ The detection of a cancer marker protein, platelet development growth factor, has also been performed using the FP.²² A few FP applications are also well known where non-aptameric ss-DNA systems have been used for protein detection.²⁵ Thus, the labeled DNA aptamers are potentially better suited, in comparison to antibodies, to implement homogeneous FP assays for detecting peptides and proteins.

Rapid detection of immunoglobulin E (IgE) is of interest in dealing with patients afflicted with allergy-mediated disorders. It is the immunoglobulin component found in the lowest levels in human serum. IgE levels, however, increase in patients afflicted with allergic asthma, atopic dermatitis, and other immune deficiency-related diseases, including AIDS. A SELEX-based high-affinity D17.4 DNA aptamer, isolated by Wiegand and co-workers,²⁶ has been utilized to assay IgE using APCE. Reasonably fast separation of the bound aptamer from the free, fluorophore-labeled DNA has been reported by the Kennedy group.¹⁶ A quartz crystal microbalance (QCM) approach using the same D17.4 ligand has also been reported by Liss and co-workers²⁷ for IgE detection with a LOD of 500 pM. More recently, a homogeneous label-free method has been adapted for sensitive, aptamer-based IgE detection using a DNA interchelating light switch complex.²⁸ Although all the above methods have demonstrated specific IgE detection with practically useful working ranges, a homogeneous FP approach using the D 17.4 DNA ligand will be a useful analytical tool considering the simplicity of the technique. It also provides a realistic possibility of aptamer-based applications for molecular diagnostics in clinical settings.

In the FP approach, the fluorophore-labeled DNA is excited by plane-polarized light and the anisotropy of the emission is calculated according to its definition.²⁹ By measuring the increase in the steady-state anisotropy of the labeled DNA aptamer when reacted with a much larger IgE, the specific detection of IgE can be performed. The applicability of serum samples for IgE detection is dependent on the binding specificity of the aptamer for IgE in the presence of possible interferences. We report on our successful attempt to use the FP approach for detecting IgE homogeneously by using the labeled D17.4 DNA aptamer. An end-labeling strategy has been successfully adapted for DNA-based FP applications.^{16,22} The binding affinity of such labeled

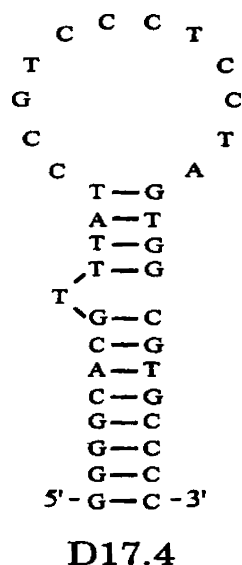


Figure 1. Predicted secondary structure of the D 17.4 IgE DNA aptamer, based on its 37-mer consensus sequence.

aptamers was not altered significantly as a result of the labeling process.¹⁶ Obtaining the greatest anisotropy change when following the DNA–target binding event, however, depends on various factors—the initial r_0 , or the limiting anisotropy of the labeled DNA ligand prior to any depolarization, the steady-state anisotropy, r , of the labeled DNA probe along with correlation of r to the motion of the DNA, and finally the size of the analyte to which the labeled DNA ligand is bound. Using the 5'-end-labeled D 17.4 ligand that has fluorescein and Texas Red conjugated as fluorophores, we have carried out the homogeneous bioanalysis of IgE under steady-state conditions based on the measurement of anisotropy changes. Binding curves, for titration experiments, of the labeled DNA with IgE clearly demonstrate the applicability of this approach. Relevant analytical figures of merit for this approach, in comparison to other analytical methods, are also reported. It is also our aim in this study to systematically assess and report the relative importance of different experimental factors such as the choice of fluorophore, labeling strategy, and temperature in order to optimize the aptamer-based FP approach.

EXPERIMENTAL SECTION

DNA aptamer D17.4, with an expected stem–loop conformation as shown in Figure 1, was used without any base modifications for the FP probe design. The base sequence for this 37-mer, isolated from SELEX trials, has been found to be 5'-GGGGCACGTT-TATCCGTCCTCCT AGTGGCGTGCCCC-3', as noted from the initial work of Wiegand and co-workers.²⁶ The three 5'-end aptamer–dye conjugates that were used for this work are shown in Figure 2. DNA aptamer with the fluorophore modifications were custom designed and purchased from Integrated DNA Technologies (Coralville, IA) in the case of conjugates **1A** (fluorescein, C6 linker) and **1C** (Texas Red, C12 linker) whereas structure **1B** (Texas Red, C6 linker) was obtained from Midland Certified Reagents Inc (Midland, TX). The unmodified DNA aptamer, used for various control experiments, was purchased from Integrated DNA Technologies. All commercial DNA samples were obtained in either HPLC or gel-purified form. Fifteen percent nondenaturing

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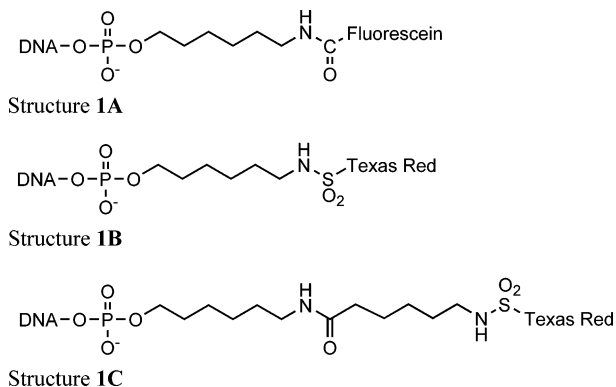


Figure 2. 1A, 5'-end fluorescein conjugate with an 8-atom tether; 1B and 1C, Texas Red conjugates with 8- and 14-atom dye linkers. DNA refers to the SELEX-based D17.4 DNA aptamer (see Figure 1).

polyacrylamide gels were used to confirm the purity of the commercial DNA samples. High-concentration DNA stock solutions were made up in water and diluted in appropriate buffer medium prior to use. Aptamer solutions were melted at 70 °C and cooled sufficiently prior to use every time, in keeping with standard ss-DNA handling practices that are designed to minimize structural misfolding. The binding buffer composition, based on the reported SELEX conditions,²⁶ was always 8.1 mM Na₂HPO₄, 1.1 mM KH₂PO₄, 1 mM MgCl₂, 2.7 mM KCl, and 138 mM NaCl and pH was adjusted to 7.4.

IgE was procured from Athens Research and Technology Inc. (Athens, GA). The protein was obtained in phosphate buffer, pH 7.2, and used without further purification. The samples were checked by the vendor's quality control experiments for >95% purity and also verified to give a single spot on immunoblotting experiments. The exact concentration of stock IgE samples was also verified using a BCA protein assay (Pierce Chemicals, Rockford, IL). The protein solutions were prepared and left on ice when not used to minimize any denaturation. Pure IgG used for aptamer specificity studies was also obtained from Athens Research and Technology Inc (Athens, GA) whereas bovine serum albumin (BSA), lysozyme, and human serum albumin (HSA) were all obtained from Sigma (St. Louis, MO).

Steady-state fluorescence experiments were all performed using a standard Quantamaster fluorometer (Photon Technology International Inc.). The spectrofluorometer is equipped with a thermostat that has a temperature control accuracy of 0.1 °C. A 6-nm slit width was usually used on both the excitation and emission slits. Both the excitation and the emission polarizers used in this work were nonmotorized Glan Thompson polarizers with medium aperture configuration (PTI Inc.). Excitation and emission wavelengths used for the fluorescein conjugate were 472 and 512 nm and 592 and 612 nm for Texas Red conjugates, respectively. Four data points, with less than 3% RSD, were collected for all anisotropy measurements, and the averaged numbers were used for further data processing. The anisotropy r of the labeled DNA was calculated based on the following definition:²⁹

$$r = \frac{(I_{VV}I_{HH}/I_{HV}I_{VH}) - 1}{(I_{VV}I_{HH}/I_{HV}I_{VH}) + 2} \quad (1)$$

where I represents the intensity of the fluorescence signal and the subscripts designate the orientation of the polarizers at the entrance and exit slits, respectively. Correction data for the background aqueous buffer solutions and for dilution factors were made to ensure that the anisotropy calculations are background subtracted and accurate. Data collection and anisotropy calculations were performed for all the experiments using Felix software for Windows OS from PTI Inc. The sample holder was a submicro quartz fluorometer cuvette (Starna Cells) with a sample volume of 100 μ L. The fluorophore-labeled DNA solutions were usually covered with aluminum foil and used the same day. All other chemicals were obtained from Sigma Chemicals.

To characterize the interaction between IgE and the dye-labeled aptamers, we used nonlinear fitting methodologies to model the observed changes in anisotropy upon titration with IgE. The relationship between the concentration of bound aptamer and steady-state anisotropy can be easily worked out if a two-state binding model is assumed. In that case, the concentration of bound aptamer is given by

$$[\text{Apt}]_b = (r - r_u)/(r_b - r_u)[\text{Apt}]_0 \quad (2)$$

where $[\text{Apt}]_b$ is the concentration of bound aptamer, r is the steady-state anisotropy, r_u is the steady-state anisotropy of the aptamer in the absence of IgE, r_b is the anisotropy at saturation levels of IgE, and $[\text{Apt}]_0$ is the total concentration of aptamer. A simple model for aptamer binding to IgE assumes full cooperativity in binding²⁸



The binding curves were fitted for two possible models—one with multiple aptamers binding to each IgE molecule and one with a single aptamer binding multiple IgE molecules. In these cases, the dissociation constants are given by $K_D = [\text{Apt}]^n[\text{IgE}]/[\text{Apt}_n\text{IgE}_n]$ and $K_D = [\text{Apt}][\text{IgE}]^n/[\text{AptIgE}_n]$, respectively. Analysis of data as a function of total IgE concentration $[\text{IgE}]_0$ and total aptamer concentration $[\text{Apt}]_0$ requires the following substitutions: $[\text{IgE}] = [\text{IgE}]_0 - n[\text{Apt}_n\text{IgE}_n]$ and $[\text{Apt}] = [\text{Apt}]_0 - n[\text{Apt}_n\text{IgE}_n]$. The final fitting equations are then

$$K_D = \frac{([\text{Apt}]_0 - n[\text{Apt}_n\text{IgE}_n])^n([\text{IgE}]_0 - [\text{Apt}_n\text{IgE}_n])}{[\text{Apt}_n\text{IgE}_n]} \quad (4)$$

and

$$K_D = \frac{([\text{Apt}]_0 - [\text{AptIgE}_n])([\text{IgE}]_0 - n[\text{AptIgE}_n])^n}{[\text{AptIgE}_n]} \quad (5)$$

for multiple aptamer molecules binding to each IgE and multiple IgE molecules binding to each aptamer, respectively.

The fitting method used for all results was nonlinear least squares. Error estimates were determined by the support plane method in which one parameter is varied while the other is fixed at values surrounding the χ^2 minimum. The F test was used to

determine the χ^2 value corresponding to one standard deviation from the minimum.³⁰

RESULTS AND DISCUSSION

Initial Steady-State Anisotropy: Effect of Labeling Strategy. Predictions and Observations. The magnitude of steady-state anisotropy changes for an FP probe during the binding process, among other factors, depends on the size of the analyte and good correlation of the measured steady-state anisotropy, r , to the global motion of the DNA to which the fluorophore is conjugated. The ability of the dye to track the global motion of the DNA and hence the characteristics of an FP probe, in turn, depends on the nature of the dye and the labeling strategy used. To predict the efficiency of the labeling strategy for the three different probes **1A**, **1B**, and **1C** used in this study (Figure 2), it is necessary to measure the steady-state anisotropy of those probes and compare them to a predicted value. The predicted value can be arrived at by knowing the size of the DNA aptamer along with certain assumptions about the impact of labeling on the DNA, as discussed below.

For estimation purposes, the r value of the labeled aptamer, calculated below, is based on the assumption that the fluorophore tracks the aptamer motion without any local motion with respect to the aptamer. The relationship between r , r_0 (limiting anisotropy of the probe), τ (dye fluorescence lifetime) and τ_{rot} (rotational correlation time) is given by the Perrin equation (eq 6), assuming a simple, monoexponential decay for the conjugated fluorophore.

$$r_0/r = 1 + \tau/\tau_{\text{rot}} \quad (6)$$

The value of r_0 is dependent on the angular displacement of the excitation transition dipole from the emission transition dipole in the labeled DNA. It was found to be 0.37, based on our concurrent time-resolved spectroscopy studies.³¹ The rotational correlation time τ_{rot} is given by the Stokes–Einstein–Debye equation.

$$\tau_{\text{rot}} = \eta V/kT \quad (7)$$

The Stokes–Einstein–Debye equation thus relates τ_{rot} to measurable solution parameters: viscosity (η), temperature (T), and solvent hydrated volume (V). The average Connolly solvent excluded volume per DNA base was calculated using Chem3D software, from CambridgeSoft, to be 231 Å³. The hydrated volume, V , for the labeled 37-mer IgE aptamer can then be calculated using the assumption that all bases contribute equally to the hydrated volume of the DNA. The value of the viscosity of water at room temperature was determined to be 897 μPa·s. The rotational correlation time of the solvated molecule was approximated to be twice the value for the anhydrous sphere as described by Yguerabide and co-workers³² for spherical macromolecules in water. Using a fluorescence lifetime of 4 ns, which is appropriate for both fluorescein and Texas Red, the expected r value of the D 17.4 aptamer in aqueous medium at room temperature was

Table 1. Observed Steady-State Anisotropy (r) Values for the Three Dye–Aptamer Conjugates **1A, **1B**, and **1C****

[DNA] (nM)	r (1A)	r (1B)	r (1C)
20	0.040	0.180	0.160
40	0.044	0.182	0.161
100	0.042	0.184	0.162

calculated to be 0.19. Though this value is an approximation based on an assumption that the DNA can be regarded as a hydrated sphere, it is nevertheless a useful value for comparison to experimental observations. The actual observed r values for the conjugates **1A**, **1B**, and **1C** at different concentrations are shown in Table 1. All the r values were found to be within a precision range of ± 0.004 . It can be noted that the r values are all different from each other and also different from the theoretically predicted value of 0.19. However, they do not vary significantly with DNA concentration, as expected. These data also suggest that the two important variables in the three dye–DNA conjugates are the linker arm length and the fluorophore itself (Figure 2). The Texas Red conjugate with the shorter linker arm, **1B**, has the highest r value of ~ 0.18 in comparison to 0.16 for the conjugate **1C** possessing the same fluorophore on the longest tether. The r value of 0.18 for **1B** is closest to the predicted anisotropy value of 0.19 whereas the value for fluorescein conjugate **1A** is the lowest at ~ 0.04 . The low r value of fluorescein conjugate **1A** is comparable to the r value obtained for a similar 33-mer system, from the work of the McGown group.³³ It is also comparable to r values obtained from other aptamer²² as well as nonaptamer-based ss-DNA systems of similar size that have used fluorescein as the fluorophore.³⁴

It is certainly surprising to note the very low steady-state anisotropy value of the fluorescein conjugate **1A** in which the dye is conjugated close to the DNA, much like the case of **1B**. This may be due to the fact that anisotropy measured in this case arises from significant segmental motion of the dye. The steady-state anisotropy corresponding to the dye rotational diffusion is usually very low due to the rapid rotational diffusion of such small-molecule fluorophores. A low anisotropy value for conjugate **1A**, especially when it is due to the local dye rotation, is unfavorable for a sensitive FP approach. The steady-state anisotropy changes, in such a case, will not be well reflective of the DNA binding properties. On the other hand, the closeness of the anisotropy values of **1C** and **1B** to the predicted anisotropy value underscores the importance of the nature of the fluorophore and relative unimportance of the dye tether length itself. Based on the r values of the three conjugates observed in Table 1, in comparison to the predicted r value of 0.19, the Texas Red conjugates **1B** and **1C** are expected to be sensitive in tracking the binding properties whereas the fluorescein conjugate **1A** is not expected to be a sensitive FP probe.

Anisotropy Changes. IgE in human serum samples is found at nanomolar levels. The binding affinity of the original, unmodified D 17.4 IgE aptamer was found to be $K_d = 9$ nM from filter binding studies.²⁶ To test the feasibility of using the three

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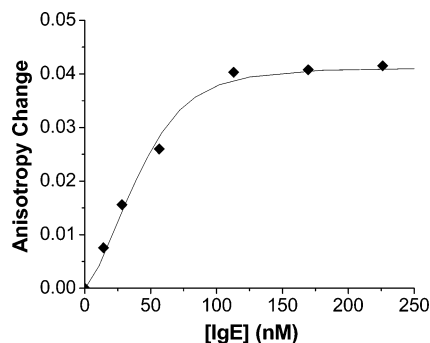


Figure 3. Titration curve of fluorescein conjugate **1A** (20nM) with increasing IgE concentrations in the standard phosphate binding buffer (see Experimental Section). Anisotropy change refers to the difference in steady-state anisotropy from the labeled DNA anisotropy in the absence of added IgE at 25 °C. The line is a nonlinear least-squares fit as described in the Experimental Section.

conjugate systems designed in this study for FP-based IgE bioanalysis, binding studies of the labeled DNA in titration experiments with different IgE concentrations were attempted. Figure 3 represents the results from the binding curve for conjugate **1A**. Conjugate **1A** (20 nM) was used in binding buffer for this experiment. As can be seen from the anisotropy change plot, the FP approach reflects the aptamer–IgE binding through measurable anisotropy changes. The assay responds to IgE levels in the range of 1–100 nM beyond which there is no further increase in anisotropy of the labeled ligand when using the 20 nM labeled DNA. The response is linear in the concentration range of 1–60 nM. The working range may be expanded using a higher concentration of aptamer although our focus was mainly in the low-nanomolar levels for sensitive IgE detection. It must be pointed out that the anisotropy change in this DNA–protein binding reaction occurs rapidly, within 1 min (data not shown). The positive control employed for this experiment confirmed the absence of anisotropy changes in the absence of IgE.

The sensitivity of the fluorescein conjugate **1A** based on anisotropy changes is, however, not as high as the r value of the labeled ligand, when apparently fully bound, i.e., about twice that of the initial value of the labeled DNA measured in the absence of any added protein. This may be explained by a significant segmental dye motion, which is already reflected in the low r value for this conjugate. As the dye segmental motion and the contribution of such a rotational component cannot be resolved and verified in the steady-state anisotropy measurements, we have approached this issue using time-correlated single photon counting spectroscopy (TCSPC) studies.³¹ In any case, as the FP method held good promise, the responses of the other conjugates for similar binding studies were also evaluated and are shown in Figure 4.

Effect of Fluorophore and Linker Arm. As can be seen from Figure 4, the FP responses for IgE detection are dependent on the conjugate used. The working ranges, as expected, are different from Figure 3 as 10 nM labeled aptamer is being used in this experiment. The Texas Red conjugate **1B** has much greater sensitivity for FP as compared to the fluorescein conjugate **1A** with a similar eight-atom linker arm. The Texas Red conjugate **1C**, possessing a much longer tether, has sensitivity comparable to **1B**. These observations are in line with our expectation based on the initial r values of the conjugates (Table 1). The higher

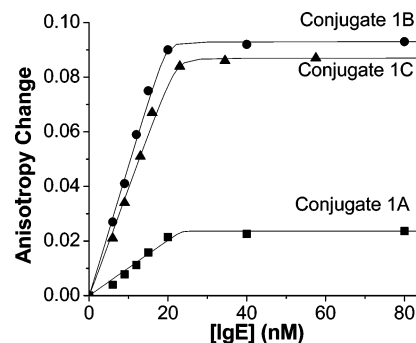


Figure 4. Comparative FP response of conjugates **1A**, **1B**, and **1C** for IgE in titration experiments where 10 nM ss-DNA aptamer was used at 25 °C. Standard phosphate binding buffer (see Experimental Section) is the assay medium used. The lines are nonlinear least-squares fits to the data as described in the Experimental Section.

sensitivity of Texas Red probes over the fluorescein probe **1A** confirms the key role of the fluorophore properties in FP probe design. The fact that both **1B** and **1C** have similar sensitivities suggests the possibility of favorable Texas Red–DNA interactions in these two probes, independent of the tether length. These interactions are probably absent in **1A**. Also the unfavorable probe characteristics observed for **1A** may arise due to a possible local fluorophore motion. Detailed analysis from our concurrent TCSPC study has validated both these possibilities.³¹ The magnitude of the local motion effect for **1A** was also quantified from the same study from which it was observed that the amplitude of the local dye motion of fluorescein dominates the global DNA motion at room temperature. Previous studies from the McGown group^{33,35} have documented similar observations. The reasoning for this observation could be that the negative charge on the fluorescein, under current experimental solution conditions, leads to a dye–DNA backbone repulsion that allows the local dye rotation.

Since the Texas Red conjugates **1B** and **1C** have higher sensitivity in detecting IgE than **1A** (Figure 4), both can be expected to have low contributions from the dye segmental motion toward their total anisotropy. This is also seen to be the case from our time-resolved spectroscopy analysis.³¹ In fact, the amplitudes of the different rotational components for **1B** and **1C** are also nearly identical. This may further explain the nearly identical binding behavior of these two probes, as seen in Figure 4. Thus, our initial expectations about the FP performance of all three probes, based on predicted anisotropy calculations, have been well justified by the observed binding data. Based on the above observations for the IgE aptamer system, we can conclude that the sensitivity of the current FP approach is determined more by the fluorophore characteristics than by the dye tether length. The working ranges of the IgE detection were, unlike sensitivity, unchanged when using these different labeled probes, as seen for example from Figure 4. Under room-temperature conditions, Texas Red conjugates are thus found to be superior FP probes for the current application.

Analysis of Binding Equilibria. Initial nonlinear fitting analysis of the anisotropy data with the two binding models described in the Experimental Section demonstrates that the model with multiple aptamer binding sites on each IgE molecule

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Table 2. Binding Parameters^a

aptamer	<i>T</i> (K)	<i>K</i> _D (nM ⁿ)	<i>n</i>	χ ²
10 nM TR12- (1C)	298	0.3 ^{+1.6_b} -0.3 _b	2.1 ± 0.2	0.08
10 nM TR6- (1B)	298	0.1 ^{+0.4} -0.1	1.9 ± 0.1	0.06
10 nM fl- (1A)	298	0.01 ^{+7.3} -0.01	2.3 ± 0.5	0.45
20 nM fl- (1A)	300	400 ⁺²²⁰⁰ -300	2.0 ^{+1.5} -0.4	0.68
20 nM fl- (1A)	310	13000 ⁺⁹⁷⁰⁰⁰ -10000	2.1 ± 0.5	0.89
8 nM TR12- (1C)	310	1500 ⁺⁸⁰⁰⁰ -1200	2.2 ± 0.5	0.18
8 nM fl- (1A)	310	25000 ⁺⁸⁰⁰⁰⁰⁰⁰ -24000	2.9 ^{+1.9} -0.8	0.23

^a Binding parameters were determined by nonlinear least squares as described in the Experimental Section. ^b Upper and lower error limits are at the one standard deviation confidence limits as determined by the *F*-test (see Experimental Section).

is not an improvement over a one-to-one binding model ($n = 1$). On the other hand, the assumption that multiple IgE molecules bind each aptamer significantly improves the fit of the data. Table 2 demonstrates that the anisotropy data is best represented by a model in which two IgE molecules bind to a single aptamer in a cooperative manner ($n = 2$). This is based on the binding parameters obtained using all three dye conjugates at different aptamer concentrations. These data are in contrast to those of Weigand and co-workers.²⁶ In that study, a filter binding assay was used to obtain a K_d of 10 nM using a monophasic model. It is difficult to ascertain the reasons for the differences between our results and theirs. The very nature of the filter binding process may disrupt the binding of the second IgE to the aptamer. Our results seem to be consistent across the different aptamers studied at room temperature and hence represent the nature of the aptamer–IgE equilibrium under homogeneous assay conditions. At room temperature, the dissociation constant is less than 10 nM to the n power. Here the power of n represents the number of IgE molecules bound to each aptamer as represented by eq 5. At higher temperatures, this value increases drastically to a value of over 1000 nM to the n power. Therefore, it seems that temperature plays a large role in the stability of the aptamer–IgE complex.

Temperature Effect on FP Response. The effect of increasing temperatures on anisotropy changes can be estimated from the Perrin equation (eq 6) and the Stokes–Einstein–Debye equation (eq 7). As the rotational correlation time, τ_{rot} , is inversely related to the temperature, the effect of increasing temperatures can result in reduced FP responses from the probes. Furthermore, viscosity itself is temperature dependent. The FP response sensitivity of each of the three conjugates under consideration may, thus, be expected to decrease with increasing temperature. In relation to this expectation, the effect of changing the assay temperature to 37 from 27 °C was studied. The binding affinity of the aptamer may vary at different temperatures since DNA is conformationally labile. However, considering the fact that the SELEX experiment²⁶ was performed at 37 °C, this was not considered to be a major issue. The results of anisotropy changes with temperature are shown in Figure 5 for conjugate **1A**.

As can be noticed, the effect of temperature is quite drastic even when the temperatures considered here were only 27 and 37 °C. The anisotropy changes are significantly lower in response

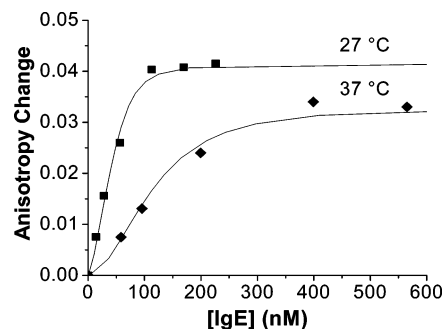


Figure 5. FP responses from fluorescein conjugate **1A** as a function of IgE concentration and temperature. 20 nM DNA aptamer was used in standard phosphate binding buffer. The lines are nonlinear least-squares fits to the data as described in the Experimental Section.

to added IgE at 37 °C. The ability of the fluorescein probe **1A** to track the binding event is seemingly lowered at 37 °C. The fact that this same probe had a low anisotropy in the absence of IgE (Table 1) and showed low sensitivity in comparison to the Texas Red conjugates at room temperature (Figure 4) suggests that segmental dye motion is even more significant at higher temperatures. Our TCSPC study could not confirm this possibility as the local dye motion for **1A** dominated the anisotropy decay even at room temperature.³¹ A similar observation has been seen reported in a previous study involving fluorophore-labeled DNA.³⁵ Thus, the temperature dependence of anisotropy changes cannot be explained by the Perrin equation alone (eq 6), which relates viscosity changes with temperature but requires consideration of the rotational dynamics of the specific probes. To confirm this effect of local dye mobility, we predicted the dye segmental motion to be less significant for both **1B** and **1C** probes at higher temperatures also, in comparison to **1A**. This is based on their superior FP probe characteristics at room temperature in comparison to **1A**, as seen from Figure 4. This prediction was also confirmed from our TCSPC analysis results.³¹ To test the effect of this prediction, the steady-state anisotropy changes for IgE detection at elevated temperature was analyzed for **1A** and **1C** (Figure 6). As expected, **1C** had higher sensitivity for FP response than **1A** when 8 nM labeled DNA aptamer was used for the experiment.

The magnitude of the sensitivity gain using **1C** instead of **1A**, as seen in Figure 6, is consistent with its 3-fold sensitivity gain observed at room temperature, as seen from Figure 4. These data establish that the assay sensitivity is clearly dependent on the probe used. The working range is again seen to depend on the aptamer concentration but not on the probe itself. The TCSPC spectroscopic studies that we have undertaken have thus helped clearly to relate the solution dynamics of the aptamer to its anisotropy characteristics and also to validate all of the temperature-dependent steady-state anisotropy observations.³¹ Choosing the Texas Red conjugates **1B** or **1C**, especially, under room-temperature conditions, can thus optimize the assay conditions for IgE bioanalysis.

Specificity Studies. The usefulness of molecular recognition-based bioanalysis for real samples will depend on the specificity of the binding process or, in effect, the impact of interfering

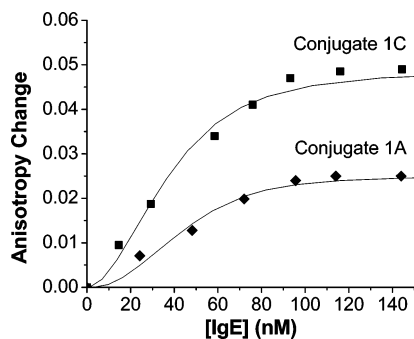


Figure 6. Comparison of FP responses of **1A** and **1C** to increasing IgE concentrations at 37 °C. FP responses were recorded in identical, standard binding buffer conditions (see Experimental Section). The lines are nonlinear least-squares fits to the data as described in the Experimental Section.

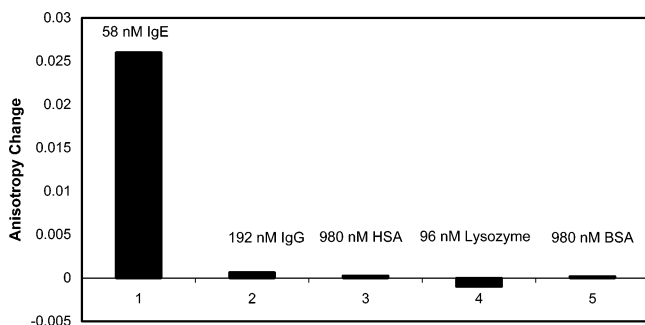


Figure 7. Specificity of 20 nM fluorescein conjugate **1A** toward potential protein interferences. All the responses are anisotropy changes upon protein addition. Measurements were made under identical solution conditions.

proteins and closely related analytes. Aptamers have had moderate success in terms of binding specificity, and the D17.4 aptamer has shown good properties in this regard. This was confirmed by a previous study using a QCM as the biosensing technique.²⁷ Liss and co-workers used different protein mixtures for specificity studies in this work and obtained encouraging responses. In our case, the measured signal is the relative FP responses of the DNA aptamer.

The interfering proteins that were tested include BSA, HSA, lysozyme, and antigenic IgG. It should be noted that the IgG is similar to IgE structurally and thus can serve as a good test protein for the aptamer binding specificity. The concentrations of the albumin proteins were chosen to be very high, as they would be in serum samples. The relative responses of the interferences, as seen in Figure 7, are insignificant in comparison to the specific binding response with IgE, in agreement with the previously reported work.^{27,28} In terms of the response to IgE binding, the cross-reactivity of the D17.4 ligand is thus seen to be less than 5% for all the interferences considered here. The relatively low aptamer cross-reactivity toward HSA, BSA, and other analytes considered here is very encouraging considering the high percentage of these serum proteins in biological samples. Although the reported data here have been obtained using the fluorescein-labeled aptamer, the binding trend is seen to be similar with conjugates **1B** and **1C**. Hence, the specificity is not found to be dependent on the dye conjugation strategies, as expected.

Effect of Cations on Aptamer Binding Properties. The binding properties of the aptamers in solution are influenced by

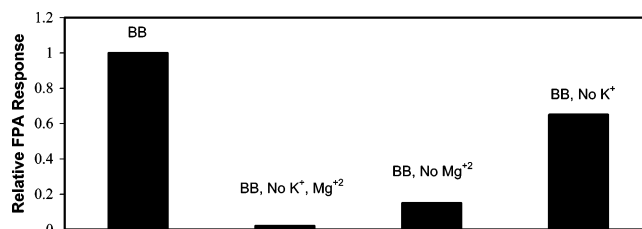


Figure 8. Effect of binding buffer (BB) composition. All FP responses are plotted in relation to a value of 1 for the specific detection of IgE, using 20 nM Texas Red conjugate **1C**.

the presence of certain cations. The binding buffer (BB) composition in the case of D17.4 aptamer²⁶ is made of 2.7 mM K⁺ and 1 mM Mg²⁺, and the flexibility of ss-DNA motif is primarily governed by the counterion interactions. Hence, the effect of the binding buffer was monitored by varying the concentration of potassium and magnesium ions. The buffer composition can have a direct effect on the binding ability of the aptamer.³⁶ Divalent cations such as magnesium ions thus typically play more than just a backbone charge compensation role and are important in determining the extended stem-loop configuration predicted in the case of the D17.4 ligand.

As seen in Figure 8, the magnesium ion concentration is quite important for efficient binding. As seen in trial 4, changing the magnesium ion concentration to the binding buffer composition levels, even in the absence of potassium ions, restores the binding ability of the DNA aptamer to 65% of the original activity. The importance of the magnesium cation is in contrast to the importance of K⁺ species in stabilizing G quartets, as seen in the case of the thrombin aptamer.³⁷ As expected, the absence of both the K⁺ and Mg²⁺ ions totally inactivates the aptamer toward any binding activity. The effect of the ions seen here is an indication of the importance of the binding buffer that was employed in all the steps of SELEX and presents a situation seen typically for different analytical applications in which aptamers are used.

Comparison to Other Methodologies. The FP method reported here presents a simple, rapid, homogeneous, and a practical method for IgE detection. As seen in this report, IgE detection can be performed at low-nanomolar levels using an end-labeled aptamer. The working range attained here is relevant considering the needs for more complex samples. Negative control experiments have shown that anisotropy changes are observed only when labeled aptamer is present and not when only the free dye, fluorescein or Texas Red, is reacted with IgE. The sensitivity of the FP method depends strongly on the fluorophore characteristics and, to a lesser extent, on the dye linker length. The specificity of aptamer binding has been found to be good. The anisotropy changes are reproducible and the limit of detection, under unoptimized conditions, attained for reliable IgE detection is 350 pM using **1C**. Careful considerations of the factors influencing the labeled DNA aptamers, as discussed in this work, can aid in designing very sensitive bioassays that can be extended to complex samples as well. The QCM²⁷ and APCE¹⁶ approaches

(36) Isom, L. M.; Sines, C. C.; Williams, L. D. *Curr. Opin. Struct. Biol.* **1999**, *9*, 298–304.

(37) Hamaguchi, N.; Ellington, A.; Stanton, M. *Anal. Biochem.* **2001**, *294*, 126–131.

are useful methods for performing IgE detection using the D17.4 ligand, but the FP approach stands out for its simplicity and user-friendly nature. The recent report²⁸ involving a chemiluminescence method for a homogeneous, label-free method to determine IgE is also particularly novel and promising. More such generic homogeneous applications can increase the bioanalytical applicability of aptamers.

CONCLUSIONS

In the current postgenomic and proteomic era, broadening the range of applications using the DNA aptamers for protein detection will be a very useful advance. Using the FP approach, it is shown here that rapid, sensitive, homogeneous IgE detection can be performed. A clear understanding of the variables when using the FP approach can help to improve the analytical figures of merit. The effects of dye labeling strategy, choice of fluorophore, temperature, and binding buffer conditions are found to influence the FP responses significantly. Texas Red is seen to be very

favorable for a sensitive readout. The importance of minimizing the segmental motion of the dye is emphasized again in this work. The role of specific cations, specifically the role of divalent magnesium ion, in determining the binding ability of the ss-DNA aptamers is also reinforced in this study. It can be expected that more homogeneous applications will be pursued actively.

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